Developmental Cell *10*, 451–459, April, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.devcel.2006.02.017

The Mouse Embryo Autonomously Acquires Anterior-Posterior Polarity at Implantation

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Summary

The earliest recognizable sign of patterning of the mouse embryo along the anteroposterior (A-P) axis is the migration of the distal visceral endoderm (DVE) toward the future anterior side. Here we report an asymmetry in the mouse embryo at an unexpectedly early stage. The gene for Lefty1, a Nodal antagonist that influences the direction of DVE migration, was found to be asymmetrically expressed in the primitive endoderm of the implanting blastocyst. *Lefty1* expression begins randomly in the inner cell mass (ICM) of the blastocyst but is regionalized to one side of the tilted ICM shortly after implantation. Asymmetric expression of *Lefty1* can be established by in vitro culture, indicating that it does not require interaction with the uterus. The asymmetric *Lefty1* expression is induced by Nodal signaling, although *Nodal* and genes for its effectors are expressed symmetrically. This asymmetry in molecular patterning of the mouse embryo pushes back the origin of the A-P body axis to the peri-implantation stage.

Introduction

Generation of polarity within an embryo is fundamental to development. In many organisms, including *Drosophila*, molecular asymmetries already present in the oocyte provide the initial polarity information for the subsequent development of body axes in the embryo. In the mouse, however, it has been thought that detectable molecular asymmetries develop well after implantation (Beddington and Robertson, 1999). Although several asymmetries in morphological features or cell fates are known to exist in mouse oocytes or preimplantation embryos, whether these asymmetries are related in any meaningful way to body axes at later stages of development has remained unknown (Rossant and Tam, 2004). Currently, no obvious molecular asymmetries have been identified in oocytes, zygotes, or preimplantation embryos, leaving the origin of body axes in the mouse unknown. Preimplantation embryo is clearly asymmetric morphologically in embryonic-abembryonic axis, but whether this axis correlates with any event in the zygote has been a subject of recent debate (Gardner, 1997, 2001; Piotrowska et al., 2001; Hiiragi and Solter, 2004).

The anteroposterior (A-P) axis is the first overt body axis that is established in the mouse embryo. The A-P axis is evidently manifested in that the visceral endoderm located at the distal tip of the embryo (distal visceral endoderm [DVE]) migrates toward the future anterior side to become the anterior visceral endoderm (AVE) (Beddington and Robertson, 1998, 1999). However, molecular asymmetries do exist before DVE movement. We have previously shown that the genes for two Nodal antagonists, Lefty1 and Cerl (Meno et al., 1996; Belo et al., 1997), are expressed asymmetrically along the future A-P axis (Yamamoto et al., 2004). This asymmetric expression is first apparent at embryonic day (E) 5.5 and is the earliest molecular asymmetry associated with mouse development identified to date. The asymmetric expression of *Lefty1* and *Cerl*, which is shifted toward the future anterior side, influences the direction of cell movement most likely by generating a directional propulsion via local inhibition of cell proliferation in the visceral endoderm. How the asymmetric expression of *Lefty1* and *Cerl* is regulated has remained unknown, however.

In this study on how asymmetric expression of *Lefty1* is induced in the visceral endoderm, we have made the surprising discovery that asymmetric *Lefty1* expression begins in the primitive endoderm shortly after the onset of implantation, which is much earlier than expected. This asymmetric *Lefty1* expression, which is induced by Nodal signaling and can be established without interaction with the uterus, pushes back the establishment of the prospective A-P body axis to the peri-implantation stage.

Results

Asymmetric Expression of *Lefty1* in Primitive Endoderm of Implanting Embryo

We have previously shown by two-color in situ hybridization that *Lefty1* is asymmetrically expressed in the visceral endoderm of E5.5 mouse embryo, before the onset of DVE cell migration (Yamamoto et al., 2004). To elucidate the asymmetric expression of *Lefty1* relative to *Hex* (*Hhex*) expression, we generated two lines of transgenic mice: one expressing the marker protein DsRed2 in *Lefty1*-expressing cells and the other expressing the marker protein Venus (Nagai et al., 2002) under the control of the DVE- and AVE-specific enhancer of *Hex* (Rodriguez et al., 2001). Embryos harboring both transgenes were examined for Venus and DsRed2 fluorescence at various stages of development.

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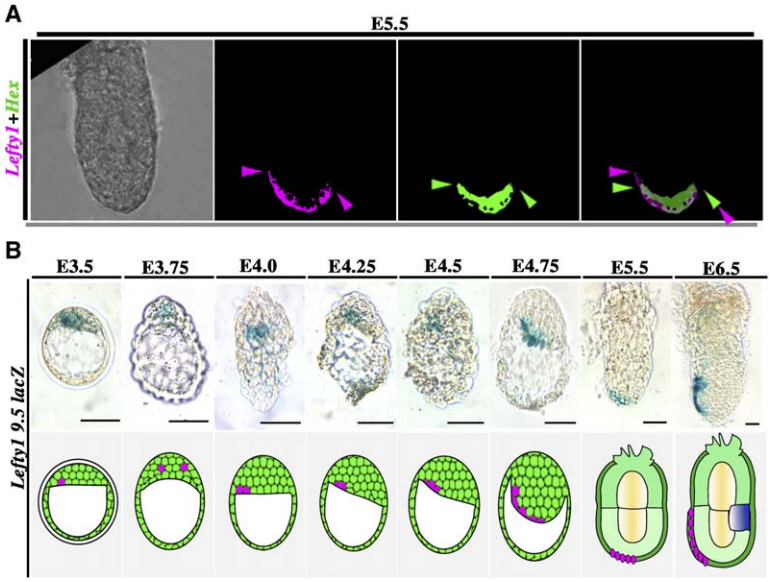

Figure 1. Asymmetric Expression of *Lefty1* Transgenes in Peri-Implantation Mouse Embryos

(A) Mouse embryos harboring two transgenes, *Lefty1-9.5 DsRed2* and *Hex-Venus*, were recovered at E5.25 or E5.5, and the fluorescence of DsRed2 (magenta) or Venus (green) was examined separately. Merged fluorescence images and phase contrast images are shown in the right and left panels, respectively. Arrowheads delineate the regions of expression of each transgene.

(B) Mouse embryos harboring the *Lefty1-9.5 lacZ* transgene were recovered at the indicated stages and stained with Xgal. Photographs of Xgal-stained embryos (upper panels) are accompanied by illustrations showing the locations of $Xgal^+$ cells (shown in magenta in lower panels). Between E4.25 and E5.5, $Xgal^+$ cells are located asymmetrically in the primitive endoderm/visceral endoderm. Embryo stages were judged as follows: E3.5, blastocyst with the zona pellucida; E3.75, hatched blastocyst with the embryonic-abembryonic axis elongated and with long and short axes; E4.0, blastocyst with more pronounced long and short axes; E4.25, implanting embryo with the tilt; E4.5, implanted embryo with a flat shape and showing formation of the decidua; E4.75, fully implanted embryo in which the ICM has grown into the blastocoel cavity. The scale bars represent 50 μm.

Venus (Hex)-positive DVE cells were located at the distal end of the embryo at E5.25 and E5.5, whereas *Lefty1*-positive cells covered a wider domain that was shifted to one side (Figure 1A; E5.25 data not shown). DsRed2-positive, Venus-negative cells were thus found on the prospective anterior side toward which the *Hex*-positive DVE cells will move (Yamamoto et al., 2004).

We investigated when asymmetric expression of *Lefty1* begins with the use of a transgenic mouse (line F32) harboring a *Lefty1-9.5 lacZ* construct that recapitulates *Lefty1* expression (Saijoh et al., 1999). Unexpectedly, *Lefty1*-expressing cells (cells stained with Xgal; 5-bromo-4-chloro-3-indoyl-β-D-galactoside) were found to be distributed asymmetrically in the primitive endoderm, being present on one side only, of embryos at E4.25 (Figure 1B). They continued to be distributed asymmetrically in the primitive endoderm at E4.5 and E4.75 (Figure 1B; embryos were staged according to their morphology).

Three lines of evidence confirmed that Xgal staining patterns obtained with the *Lefty1-9.5 lacZ* transgene reflected genuine *Lefty1* expression. First, we generated a transgenic mouse line (B1) that harbors a 250 kb bacterial artificial chromosome (BAC) clone (RP23-390I1) of *Lefty1*, with exon 1 of *Lefty1* being replaced by *lacZ*. Xgal staining of embryos of this line again revealed transgene expression on one side of the primitive endoderm at E4.5 and of the DVE at E5.5 (Figure 2A). Second,

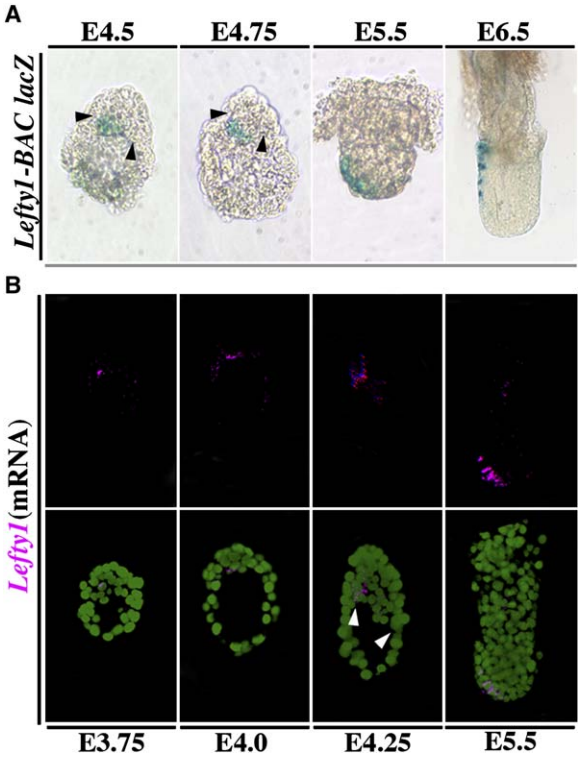

Figure 2. Asymmetric *Lefty1* Expression as Revealed by a *Lefty1* Transgene and by In Situ Hybridization

(A) Mouse embryos harboring a *Lefty1-BAC lacZ* transgene were recovered at the indicated stages and stained with Xgal. Arrowheads indicate junctions between the primitive endoderm and the trophectoderm.

(B) Fluorescence in situ hybridization of whole-mount preparations of mouse embryos at the indicated stages with a probe specific for *Lefty1* mRNA (magenta). Nuclei were counterstained with the dye YOYO-1 (green). The distribution of *Lefty1* mRNA was similar to that of Xgal staining in *Lefty1-lacZ* transgenic embryos. At E4.2, for example, when the tilt of the ICM is apparent, *Lefty1* mRNA was localized to the "upper" side of the tilt, as were $Xgal^+$ cells in *Lefty1-lacZ* transgenic embryos. Arrowheads indicate junctions between the primitive endoderm and the trophectoderm.

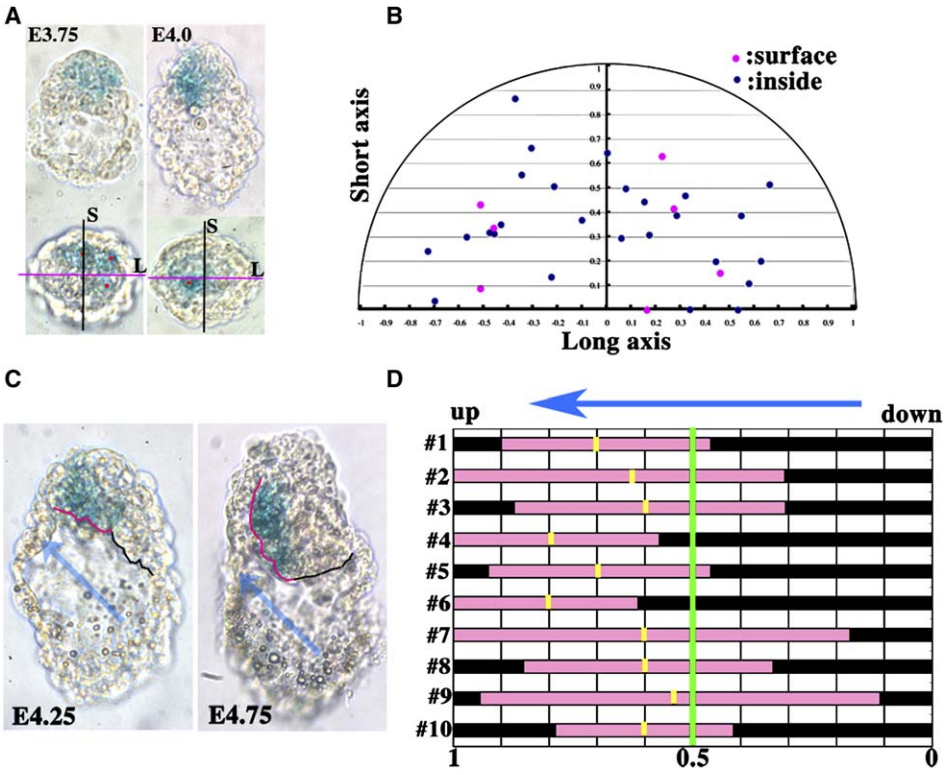

Figure 3. Localization of *Lefty1*-Expressing Cells in the Mouse Embryo

(A) Transgenic mouse embryos harboring the *Lefty1-0.7 lacZ* transgene were recovered at the indicated ages and stained with Xgal. The positions of Xgal$^+$ cells were examined by observing embryos from various angles. Xgal$^+$ cells were detected inside of the ICM at E3.75 and at the surface of the ICM facing the blastocoel at E4.0. In some embryos at these stages, Xgal$^+$ cells were found in both locations. Lateral views of representative embryos are shown in the upper panels, whereas the long (L) and short (S) axes in the view from the abembryonic pole are indicated in the lower panels. The red dots indicate the center of Xgal-stained cells.

(B) Summary of the positions of Xgal$^+$ cells relative to the long and short axes of embryos at E3.75 (n = 11) or E4.0 (n = 8) determined as in (A). The whole ICM was partitioned into four quadrants by the long and short axes, and the position of the Xgal-stained cells was scored by a Cartesian (x, y) coordinate with reference to the long (x) and short (y) axes. For presentation, the cells on either side of the long axis are mapped to the hemisphere on the positive (y = 0 to +1) side of the long axis. Blue dots and pink dots denote Xgal$^+$ cells located inside or at the surface of the ICM, respectively.

(C) *Lefty1-0.7 lacZ* transgenic embryos at E4.25 and E4.75 stained with Xgal. The tilt of the ICM is indicated by the blue arrow. Xgal$^+$ and Xgal$^-$ populations of the primitive endoderm are indicated by red and black lines, respectively.

(D) Summary of the locations of Xgal$^+$ cells along the tilt of *Lefty1-0.7 lacZ* embryos (#1 to #10) at E4.25. Red and black bars represent Xgal$^+$ and Xgal$^-$ cells, respectively. The midpoint of the Xgal$^+$ region is indicated by the yellow bar.

a transgenic mouse line (*Lefty1-0.7 lacZ*) in which *lacZ* expression is regulated by the minimal 0.7 kb enhancer fragment of *Lefty1* manifested asymmetric Xgal staining at E4.5 (see below). Third, *Lefty1* mRNA was detected by fluorescence in situ hybridization on one side of the primitive endoderm of wild-type embryos between E4.25 and E5.5 (Figure 2B).

Localization of *Lefty1*-Expressing Cells in Peri-Implantation Embryos

Expression of the *Lefty1-9.5 lacZ* and *Lefty1-0.7 lacZ* transgenes was first detected in the blastocyst at E3.5 (Figure 1B; also see below). Expression of *Lefty1* likely begins at E3.5 because most (70%) of the transgenic embryos remained Xgal negative at this time. In the Xgal-positive embryos, stained cells were apparent in the inner cell mass (ICM), but it was not possible to determine their precise location because obvious topological features are limited at this stage. However, Xgal$^+$ cells were found at both the periphery or central region of the ICM and at either the deep or superficial location in the ICM (Figure 1B; see below). These findings suggest that *Lefty1*-expressing cells are randomly distributed in the ICM of the E3.5 blastocyst.

Mouse embryos between E3.75 and E4.0 are bilaterally symmetric and exhibit a long axis and a short axis (Smith, 1980, 1985; Gardner, 1997). The location of *Lefty1*-positive cells relative to the short axis and long axis was determined in *Lefty1-0.7 lacZ* embryos. At E3.75, when *Lefty1* expression was detected inside of the ICM (Figure 3A), embryos contained an average of three Xgal$^+$ cells (n = 10 embryos), and these cells showed no preferential localization. For instance, Xgal$^+$ cells were detected in equal numbers in the quadrant on either side of the short axis when viewed from the abembryonic pole (Figure 3B). In embryos at E4.0 (Figure 3A), *Lefty1*-expressing cells were mainly found near the blastocoelic surface of the ICM, where prospective primitive endoderm cells reside (Rossant et al., 2003). The Xgal$^+$ cells of each embryo were in a cluster but are still randomly distributed relative to both the long

and short axes (Figure 3B; $p = 0.471 >> 0.05$ according to Pearson's correlation coefficient test).

At E4.25, the mouse embryo develops a transient asymmetry, which is highlighted by the tilting of the ICM along the long axis of the implanting blastocyst (Smith, 1980, 1985). It has been suggested that the tilt of the ICM may mark the orientation of the future A-P axis (Smith, 1985; Gardner et al., 1992). The lopsided orientation of the ICM provides a morphological indication of the polarity of the long axis, which can be used as a reference landmark for determining the location of $Xgal^+$ cells in the ICM. Cells positive for Xgal staining were located on the "upper" side (the side closer to the "upper" edge) of the tilted ICM in all ten embryos examined (Figures 3C and 3D). It is worth noting again that *Lefty1* mRNA was also detected on the upper side of the primitive endoderm at E4.25 (Figure 2B). *Lefty1*-expressing cells were similarly localized to the upper side of the tilted ICM at E4.5 (Figures 1B and 2A) and E4.75 (Figures 1B, 2A, and 3C). Together, these results suggest that *Lefty1*-expressing cells are initially distributed randomly in the ICM at E3.5, but by E4.25 they become localized to the primitive endoderm located closer to one pole of the long axis of the implanting blastocyst.

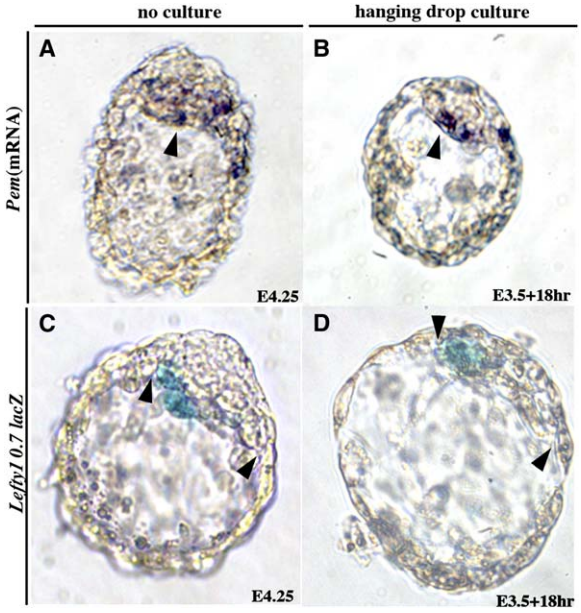

Figure 4. Asymmetric Expression of *Lefty1* in Cultured Mouse Embryos

(A and B) Wild-type embryos were recovered at E3.5 and cultured in hanging drops for 18 hr after hatching as described in Experimental Procedures. The cultured embryo (B) was examined by in situ hybridization for expression of *Pem*, a marker for the primitive endoderm. Noncultured embryos at E4.25 (A) were examined for comparison. Arrowheads in (A) and (B) indicate *Pem*-expressing cells in the primitive endoderm.

(C and D) Transgenic embryos harboring *Lefty1-0.7 lacZ* were recovered at E3.5 and cultured in hanging drops for 18 hr after hatching. The cultured embryos were stained with Xgal to detect expression of *Lefty1-0.7 lacZ* (D). Noncultured embryos at E4.25 (C) were examined for comparison. Arrowheads in (C) and (D) indicate junctions between the primitive endoderm and the trophectoderm.

Asymmetric *Lefty1* Expression in the Primitive Endoderm Does Not Require Interaction with the Uterus

The E4.25 embryo that displays asymmetric *Lefty1* expression (Figure 3C) is loosely attached to the uterus within the implantation chamber but is not fully implanted. It has been suggested that the uterus may provide positional information to the mouse embryo (for a review, see Tam et al., 2001), and therefore the asymmetric *Lefty1* expression in the primitive endoderm could be dependent on the interaction of the embryo with the uterine tissues at implantation.

To test the role of implantation in the establishment of asymmetry of *Lefty1* expression domain, preimplantation embryos were cultured in vitro over the implantation period without being attached or exposed to the uterine environment. Embryos harboring *Lefty1-0.7 lacZ* were recovered at E3.5 and cultured in hanging drops until they developed to a stage equivalent to E4.25. *Pem*, a marker for the primitive endoderm (Lin et al., 1994), was expressed in the cultured embryos (Figure 4B), showing that the primitive endoderm has developed normally. In these embryos, *Lefty1* was expressed asymmetrically in the primitive endoderm. In embryos that formed a tilted ICM (7/13 embryos; Figure 4D), $Xgal^+$ cells were localized to the upper side of the primitive endoderm (all 7 embryos). Even in the remaining 6 embryos that did not display an obvious tilt of the ICM, $Xgal^+$ cells were asymmetrically localized to one side of the primitive endoderm (5/6 embryos: data not shown). These results strongly suggest that asymmetric expression of *Lefty1* in the primitive endoderm is acquired autonomously in the implanting embryo and does not require any interaction with the uterus.

Asymmetric Expression of *Lefty1* Is Induced by Nodal Signaling

To investigate the mechanism by which asymmetric *Lefty1* expression is achieved in the primitive endoderm and AVE, we searched for the transcriptional enhancer responsible for such expression. A series of 5′ deletion fragments derived from the 9.5 kb upstream region of *Lefty1* was examined with a transient transgenic assay for enhancer activity that gives rise to *lacZ* expression in the AVE at E6.5. Testing of the various *lacZ* constructs localized such enhancer activity to a 0.7 kb region positioned between 8.5 and 7.8 kb upstream of the transcription start site (Figure 5A). The nucleotide sequence of this 0.7 kb region (Figure 5B) is highly conserved among mouse, rat, and human and contains a pair of binding sequences for FoxH1 (TGTGGATT, TGTGGATT), a transcription factor that mediates Nodal signaling (Whitman, 1998; Saijoh et al., 2000). A transgene in which both putative FoxH1 binding sites in the 0.7 kb region are mutated (*Lefty1-0.7Fm lacZ*) failed to give rise to Xgal staining in the AVE at E6.5 (Figures 5A and 6B), suggesting that the enhancer activity depends on Nodal signaling. We designated this enhancer APE (*a*symmetric *p*rimitive endoderm enhancer).

The 0.7 kb region of *Lefty1* containing the two FoxH1 binding sites was not only active in the AVE at E6.5 but also gave rise to asymmetric Xgal staining in the primitive endoderm between E4.25 and E4.75 (Figures 5A and 6A). The *Lefty1-0.7Fm lacZ* construct did not exhibit the latter activity (Figures 5A and 6B), indicating that

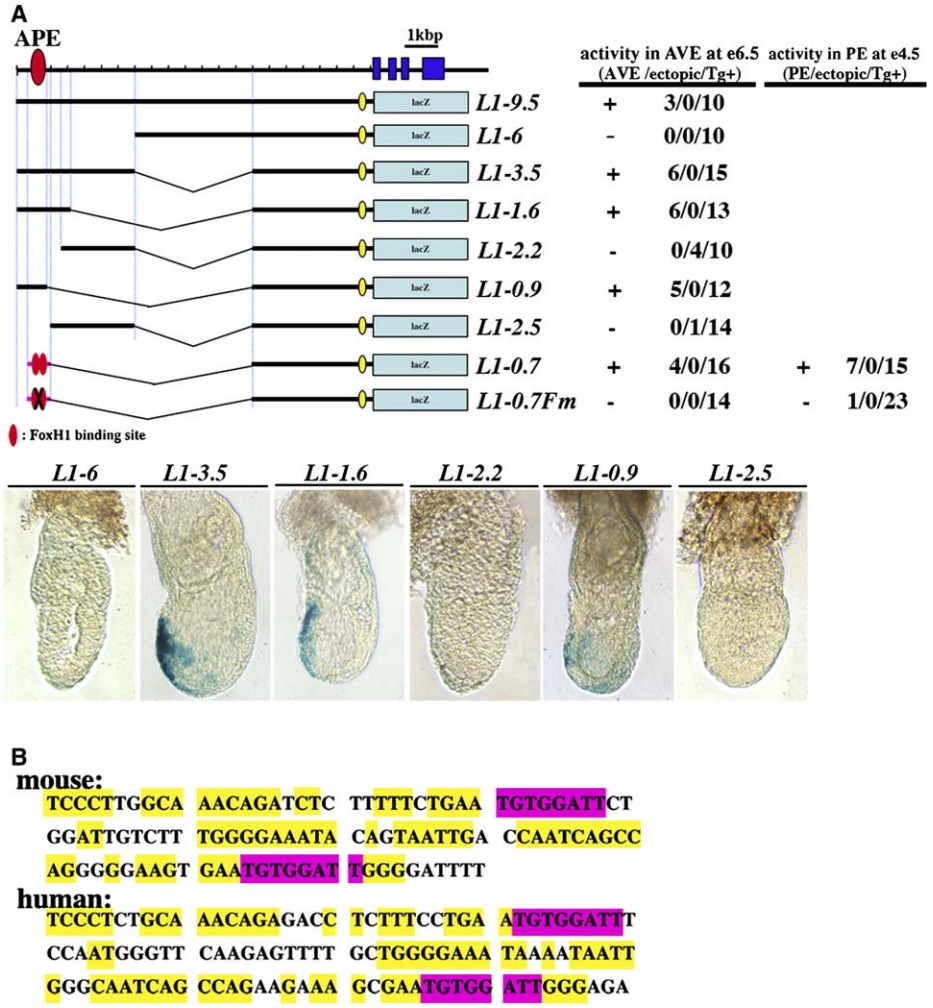

Figure 5. Mapping of the Enhancer Responsible for Asymmetric *Lefty1* Expression

(A) Nine *lacZ* constructs containing portions of the 9.5 kb upstream region of *Lefty1* (L1) were tested with a transient transgenic assay for their ability to give rise to *lacZ* expression in the AVE at E6.5. Representative Xgal-stained embryos harboring the indicated constructs are shown in the lower panels. Results are summarized above as number of embryos with Xgal staining in the AVE/number of embryos with ectopic Xgal staining/total number of transgenic embryos. For two constructs (*Lefty1-0.7 lacZ* and *Lefty1-0.7Fm lacZ*), transgenic embryos were also recovered at E4.5 and examined for expression of *lacZ* in the primitive endoderm (PE); results are summarized as for analysis of expression in the AVE. The enhancer (designated APE) that confers *lacZ* expression in the AVE was mapped to the 0.7 kb region of *Lefty1* in *Lefty1-0.7 lacZ*. Two FoxH1 binding sequences present in this 0.7 kb region are indicated by red ovals. Yellow ovals indicate the transcription start site of *Lefty1* and the dark blue boxes indicate exons of *Lefty1*.

(B) Nucleotide sequences of the 0.7 kb region of mouse *Lefty1* that contains the APE and their conservation in human *LEFTY1*. The conserved FoxH1 binding sequences and other conserved sequences are shown in magenta and yellow, respectively.

asymmetric *Lefty1* expression at this time is also induced by Nodal signaling. We also examined expression of the *Lefty1-9.5 lacZ* transgene in *Foxh1*$^{-/-}$ embryos. The transgene was inactive between E3.5 and E6.5 in the mutant embryos (Figure 6C). These results thus indicated that asymmetric *Lefty1* expression in the primitive endoderm and the AVE is induced by Nodal signaling acting at the FoxH1-dependent enhancer APE.

Lack of Asymmetry in the Expression of *Nodal* and of Genes for Nodal Effectors

Given the dependence of the identified *Lefty1* enhancer on Nodal signaling, we examined the expression of *Nodal* as well as that of genes for components of the Nodal signaling pathway, including FoxH1 and Cripto, in the mouse embryo between E3.5 and E4.75 (Figure 7). Both *Nodal* and *Foxh1* were expressed in the ICM at E3.5 as well as in both the epiblast and the primitive endoderm between E4.25 and E4.75. *Cripto* was also expressed in the ICM at E3.5 and in the epiblast between E4.25 and E4.75, but *Cripto* expression was not evident in the primitive endoderm during the latter period. *Nodal*, *Foxh1*, and *Cripto* are thus all expressed relatively uniformly between E3.5 and E4.75, with no evidence of asymmetry. The expression of *Cripto* in the ICM of E3.5 embryo indicates that some ICM cells may coexpress both *Cripto* and *Lefty1*, which is consistent with the concept that the initiation of *Lefty1* expression may be dependent on Nodal signaling. However, the absence of *Cripto* expression in the primitive endoderm

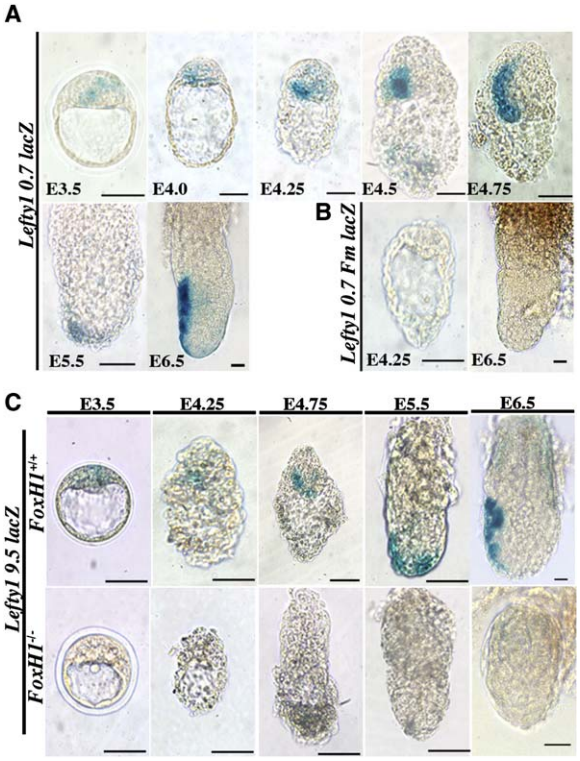

Figure 6. Asymmetric *Lefty1* Expression in the Primitive Endoderm Induced by Nodal Signaling

(A) Embryos of a permanent transgenic mouse line harboring the *Lefty1-0.7 lacZ* transgene were recovered at the indicated stages and stained with Xgal. The scale bars represent 50 μm.

(B) Transient transgenic embryos harboring *Lefty1-0.7Fm lacZ* were recovered at E4.25 or E6.5 and stained with Xgal. The scale bars represent 50 μm.

(C) Expression of the *Lefty1-9.5 lacZ* transgene in *Foxh1*$^{+/+}$ or *Foxh1*$^{-/-}$ embryos at the indicated stages. The scale bars represent 50 μm.

between E4.25 and E4.75 (Figure 7) suggests that Nodal signaling may not be required for sustaining *Lefty1* expression in the early postimplantation embryo.

Discussion

We have identified the earliest known molecular patterning in the mouse embryo. Our results thus show that the mouse embryo is already patterned along the future A-P axis very early during peri-implantation development. In addition, the asymmetric expression of *Lefty1* in the primitive endoderm was shown to be induced by Nodal signaling, although Nodal and its effectors are expressed without asymmetry. Furthermore, we found that establishment of asymmetric *Lefty1* expression does not require any interaction of the embryo with the uterus.

Origin of A-P Polarity

The asymmetric *Lefty1* expression in the implanting embryo pushes back the origin of the A-P body axis of the mouse embryo as early as peri-implantation. Then, what is the origin of A-P polarity? It must exist within a preimplantation embryo because asymmetric *Lefty1* expression can be established without implantation. The fate of *Lefty1*-expressing cells in the peri-implantation embryo must be determined in the future, but the *Lefty1*-expressing cells in the blastocyst may contribute to *Lefty1*-positive cells in the primitive endoderm at later stages. If this is the case, the origin of A-P polarity can be traced back to the *Lefty1*-positive cells in the blastocyst. The positions of *Lefty1*-expressing cells in the ICM suggest that *Lefty1*-expressing cells may arise randomly within the ICM. In view of that some cells in the ICM already show differential expression of *Gata6* which may be fated to become the primitive endoderm (Rossant et al., 2003), it is likely that the *Lefty1*-expressing cells are the progenitor of the primitive endoderm of the postimplantation embryo.

FoxH1 binding sequences located within the asymmetric enhancer (APE) were essential for the asymmetric *Lefty1* expression. Formally, two mechanisms are possible. The FoxH1 binding sequences alone may be responsible for inducing *Lefty1* expression, as has been reported for the left-right asymmetric enhancers of *Nodal* and *Lefty2* genes (Saijoh et al., 2000). Alternatively, while the FoxH1 binding site may be a component of the APE, there may be some other transcription factor recognition sequence(s) that regulates the asymmetry of *Lefty1* expression. Because FoxH1 can mediate not only Nodal signal but also signals of other TGFβ members including TGFβ and Activin (Whitman, 1998), other TGFβ members may also play a role in *Lefty1* regulation. In particular, GDF3 may be a good candidate because mutant mice lacking Gdf3 manifest A-P patterning defects (Chen et al., 2005). In fact, *Gdf3* is also expressed in peri-implantation mouse embryos without asymmetry (see Figure S1 in the Supplemental Data available with this article online).

Lefty1 expression in the blastocyst was confined to two or three cells within the ICM. Similarly, *Lefty1* is expressed only in the primitive endoderm on one side of the peri-implantation embryo. However, *Nodal* and the genes for Nodal effectors such as FoxH1 and Cripto are all expressed symmetrically. The absence of *Cripto* expression in the primitive endoderm between E4.25 and E4.75 suggests that *Lefty1* expression is initiated at E3.5 by Nodal signaling and is maintained in the primitive endoderm lineage by a Nodal-independent mechanism. A similar mechanism regulates asymmetric expression of *Pitx2* at the early somite stage. Asymmetric *Pitx2* expression is initiated by Nodal signaling acting at FoxH1 binding sites but is maintained in the absence of Nodal in a manner dependent on an Nkx2.5 binding sequence located near the FoxH1 binding sites (Shiratori et al., 2001). Nodal signaling might be required only for the initiation of *Lefty1* expression. Alternatively, Cripto protein produced in the epiblast may act on the adjacent visceral endoderm as Cripto can act in cell-autonomous as well as nonautonomous manners (Chu et al., 2005).

However, given that expression of *Nodal*, *Foxh1*, and *Cripto* is uniform in the ICM at E3.5, how is *Lefty1* expression activated in some cells but not in others? It is possible that an unknown factor required for Nodal signaling is expressed in a specific and asymmetric manner. Crosstalk from other signaling pathways may also be involved (Ang and Constum, 2004). For example, expression of *Hex* in AVE (and most likely *Lefty1* in DVE) is

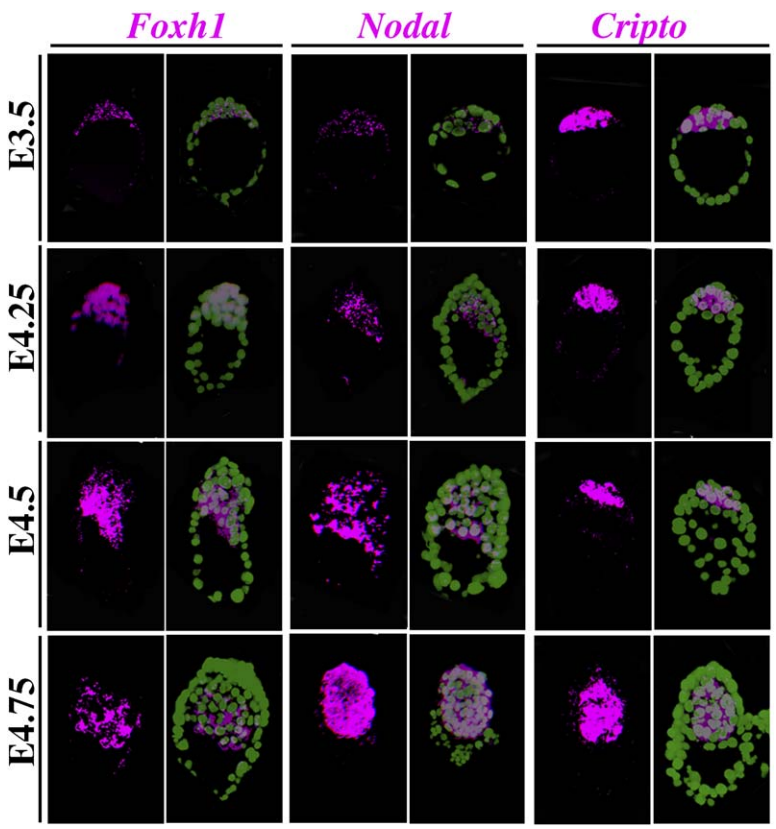

Figure 7. Lack of Asymmetry in the Expression of *Nodal*, *Foxh1*, and *Cripto* in Mouse Embryos between E3.5 and E4.75

Expression of *Nodal*, *Foxh1*, and *Cripto* in mouse embryos was examined at the indicated stages by fluorescence in situ hybridization. Signals for the mRNA of each gene are shown in magenta, with YOYO-1 fluorescence in nuclei being shown in green.

repressed by signals from the extraembryonic ectoderm (Rodriguez et al., 2005). Similarly, it is possible that signals from the polar trophectoderm regulate asymmetric *Lefty1* expression. Although it is unknown whether the polar trophectoderm is molecularly polarized, it is interesting to note that a flow of polar trophectoderm to the mural region is polarized, such that more net flow takes place in one quadrant of the blastocyst than others (Gardner, 1996).

Alternatively, an autonomous mechanism may exist that stochastically induces *Lefty1* expression in a cell, in turn resulting in repression of *Lefty1* expression in other cells. One can envisage a mechanism resembling lateral inhibition. Interestingly, Nodal and Lefty constitute a reaction-diffusion system (T. Nakamura, N. Mine, E. Nakaguchi, M.Y., Y.S., C.M., and H.H., unpublished data), a theoretical model that is able autonomously to generate various patterns during development (Turing, 1952). Stochastic activation of gene activity coupled with a reaction-diffusion system comprised of Nodal and Lefty1 may be sufficient to generate such an asymmetry that predisposes A-P patterning of the periimplantation embryo. Clarification of this issue may be achieved by further analysis of the mechanism by which the responsible enhancer of *Lefty1* (APE) is activated in the blastocyst.

Implantation Does Not Provide the Positional Cue for A-P Polarity

The uterus provides various signals to an implanting embryo that are required for postimplantation development (Tam et al., 2001; Rossant and Tam, 2004). Signaling by uterus-derived factors such as HB-EGF (heparin binding epidermal growth factor-like growth factor) is known to be activated within the embryo at the site of implantation (Das et al., 1994). Endometrial cells at the site of implantation express several signaling molecules (Paria et al., 2001). *LEFTY2* (*EBAF*) is also expressed in the endometrial cells of the human uterus (Kothapalli et al., 1997). Indeed, the axes of an embryo and the anatomic axes of the uterus appear to be related. Alignment of the A-P axis of the embryo with the transverse plane of the uterine horn (Smith, 1980) may be achieved by interaction between an implanting embryo and the uterus. Rare instances have been described of preimplantation mouse embryos developing in culture to a stage resembling the early somite stage (Hsu, 1979; Libbus and Hsu, 1980). However, the significance of these findings remains unknown, in part because the embryos were kept attached to the surface of a plastic dish. It has thus been generally believed that the uterus influences axis development in the embryo.

Our experiments involving the culture of mouse embryos in hanging drops have now shown that interaction with the uterus is not necessary for establishment of asymmetric *Lefty1* expression in the primitive endoderm. The mouse embryo thus appears capable of establishing initial A-P polarity autonomously, without positional information relative to the uterine axes. These results appear consistent with the recent finding that A-P polarity of the gastrulating mouse embryo at E6.0 is not related to any specific anatomic axis of the uterus (Mesnard et al., 2004). It is interesting to note that in marsupial mammals, axis formation occurs before implantation (reviewed by Selwood, 2001; Eakin and Behringer, 2004). Thus, marsupial embryos implant

late in development, such that gastrulation takes place well before any intimate physical interaction is established between the conceptus and the uterus. Therefore, the factors required for axis formation in marsupials are intrinsic to the embryo. Our findings in mouse embryos suggest an evolutionarily conserved mechanism common to metatherian and eutherian mammals whereby axis determination is an embryo-autonomous process.

Experimental Procedures

Generation of Transgenic Mice

A transgenic mouse line (F32) harboring *Lefty1-9.5 lacZ* (a *lacZ* reporter that contains the 9.5 kb upstream region of *Lefty1*, including the transcription start site) was established previously (Saijoh et al., 1999). The *lacZ* transgene of F32 mice recapitulates *Lefty1* expression in the floor plate and left lateral plate at the early somite stage. The transgene *Lefty1-9.5 DsRed2* was constructed similarly, with *lacZ* being replaced by *DsRed2mit* (BD Biosciences). The *Hex-Venus* transgene contains the 1.2 kb upstream region of *Hex* (as the minimal promoter), the 1 kb region from intron 1 of *Hex* that has been shown to contain the DVE/AVE-specific enhancer (Rodriguez et al., 2001), and *Venus* (Nagai et al., 2002). The *Lefty1-BAC lacZ* transgene contains the *Lefty1* BAC RP23-390I1, with exon 1 of *Lefty1* being replaced by *lacZ*. The highly efficient phage-based recombination system for *Escherichia coli* (Copeland et al., 2001) was used to construct the recombinant BAC clone. BAC DNA was prepared for microinjection as described (Gong et al., 2003). Transgenic mice were generated as described previously (Saijoh et al., 1999). F32 mice were crossed with *Foxh1* mutant mice (Yamamoto et al., 2001) to examine *lacZ* transgene expression in the absence of FoxH1.

Recovery of Peri-Implantation Mouse Embryos

Preimplantation embryos at stages up to E4.0 were recovered by flushing the uterus. Embryos at E4.2 and later were dissected out from the uterus. The embryos were staged on the basis of their morphology and their manner of interaction with the uterus.

Xgal Staining and In Situ Hybridization

Transgenic embryos were stained with Xgal as described previously (Saijoh et al., 1999). Fluorescence in situ hybridization was performed with a method designed for peri-implantation embryos (Strumpf et al., 2005; also see http://www.sickkids.on.ca/rossant/protocols/doubleFluor.asp). Nuclei were stained with YOYO-1 (Molecular Probes).

In Vitro Culture of Preimplantation Embryos in Hanging Drops

Mouse embryos harboring *Lefty1-0.7 lacZ* were recovered at E3.5, cultured in KSOM medium (Lawitts and Biggers, 1993) until they hatched, transferred to hanging drops comprising Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, and incubated for an additional 18 hr. To determine optimal conditions for hanging drop culture, we tested four different lots of fetal bovine serum that were intended for culture of mouse embryonic stem cells and one lot of rat serum prepared in house for culture of mouse embryos. One lot of fetal bovine serum (Hyclone; catalog no. SH30070.02, lot no. AQC23292) that supported the development of embryos with a relatively normal morphology as well as expression of *Pem* in the primitive endoderm was used in this study. Incubation of embryos for a longer time (24 hr) after hatching did not improve development. Under the optimal conditions (incubation in hanging drops containing KSOM until hatching followed by further incubation in hanging drops in DMEM plus 20% fetal bovine serum for 18 hr), embryos developed to a stage equivalent to E4.25 in terms of morphology (in particular, the tilt) and the expression pattern of *Pem*. The time of hatching was variable among the embryos in the first phase of culture in KSOM.

Mapping of the AVE-Specific Enhancer of *Lefty1*

F32 transgenic embryos showed specific expression of *lacZ* in the AVE between E5.5 and E6.5. To map the enhancer responsible for such expression, we generated various smaller DNA fragments from the 9.5 kb upstream region of *Lefty1* and tested them for enhancer activity with a transient transgenic assay, as described previously (Saijoh et al., 1999). All constructs contained the minimal promoter of *Lefty1* (the 3 kb upstream region). Transgenic embryos were recovered at E6.5 (to detect enhancer activity in the AVE) or at E4.5 (to detect asymmetric *lacZ* expression in the primitive endoderm). In *Lefty1-0.7Fm*, two FoxH1 binding sequences (TGTGGATT) were changed to TGTGGcag and TGTGGccc.

Supplemental Data

Supplemental Data include one figure and are available at http://www.developmentalcell.com/cgi/content/full/10/4/451/DC1/.

Acknowledgments

We thank Y. Yamanaka for advice on fluorescence in situ hybridization, N. Copeland for sharing the BAC recombination system, S. Gong for advice on BAC transgenesis, A. Miyawaki for *Venus*, S. Yamanaka for a *Gdf3* probe, P. Tam for comments including advice on hanging drop culture, as well as K. Mochida, Y. Ikawa, K. Miyama, S. Ohishi, and K. Yamashita for technical assistance. This work was supported by a grant from CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation.

Received: January 2, 2006
Revised: February 21, 2006
Accepted: February 23, 2006
Published: April 3, 2006

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